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Ninth day of April 2003

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Regulation 3.2

Medvet Science Pty Ltd

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A Method of Modulating Cellular Activity""

The invention is described in the following statement:

A METHOD OF MODULATING CELLULAR ACTIVITY

FIELD OF THE INVENTION

The present invention relates generally to a method of modulating cellular activity and to agents for use therein. More particularly, the present invention provides a method of modulating cellular activity by modulating phosphorylation of sphingosine kinase and, thereby, its activation. In a related aspect, the present invention provides a method of modulating sphingosine kinase functional activity via modulation of its phosphorylation and agents for use therein. The present invention still further extends to sphingosine kinase variants and to derivatives, analogues, chemical equivalents and mimetics thereof exhibiting reduced and/or ablated capacity to undergo phosphorylation. The method and molecules of the present invention are useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate cellular and/or sphingosine kinase functional activity. The present invention is further directed to methods for identifying and/or designing agents capable of modulating sphingosine kinase phosphorylation.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Sphingosine kinase catalyses the formation of sphingosine 1-phosphate (S1P), a lipid messenger that plays important roles in a wide variety of mammalian cellular processes [1,2]. S1P is mitogenic in various cell types and triggers a diverse range of important regulatory pathways including; mobilisation of intracellular calcium by an inositol

triphosphate independent pathway [3], activation of phospholipase D [5], inhibition of c-Jun N-terminal kinase (JNK) [6], inhibition of caspases [6], adhesion molecule expression [7], and stimulation of DNA binding activity of NF-κB [8] and transcription factor activator protein-1 (AP-1) [9].

In addition to its role in cellular proliferation and survival, S1P appears to have other functions in the cell. For example, recent studies have shown that S1P is an obligatory signalling intermediate in adhesion molecule expression of vascular endothelial cells [7], suggesting a likely role in inflammation and atherosclerosis.

Cellular levels of S1P are largely mediated by the activity of sphingosine kinase, and to a lesser extent by its degradation by S1P lyase [12] and S1P phosphatase [13] activities. Basal levels of S1P in the cell are generally low [14], but can increase rapidly and transiently when cells are exposed to various mitogenic agents. This response is a direct consequence of an increase in sphingosine kinase activity in the cytosol and can be prevented by the addition of sphingosine kinase inhibitors. This places sphingosine kinase, and its activation, in a central and obligatory role in mediating the observed effects attributed to S1P in the cell. However, at present almost nothing is known of the mechanism(s) leading to sphingosine kinase activation.

Sphingosine kinase can be very rapidly activated by wide variety of cell agonists. While the response differs between cell types, these stimuli include TNFα [7,15](Fig 1), platelet-derived growth factor [16], epidermal growth factor [17], nerve growth factor [18], vitamin D3 [19], phorbol esters [15,20], acetylcholine (muscarinic agonists) [21], and crosslinking of the immunoglobulin receptors FcεR1 [22] and FcγR1 [23]. In all cases this sphingosine kinase activation increases the V_{max} of the reaction while leaving the substrate affinities (K_m) unaltered.

The molecular mechanisms that couple these agonists to activation of sphingosine kinase activity remain largely unknown. Accordingly, there is a need to elucidate these mechanisms and to develop methods of regulating cellular activities via regulation of the

sphingosine kinase signalling pathways.

In work leading up to the present invention, the inventors have determined that phosphorylation of sphingosine kinase is essential for its activation. Further the inventors have identified the phosphorylation sites on the sphingosine kinase molecule. Further, it has been determined that phosphorylation of sphingosine kinase is performed by a proline-directed protein kinase, more particularly ERK2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Phosphorylation of hSK1 parallels its activation. TNFα simulation of hSK1-transfected HEK293T cells resulted in an *in vivo* phosphorylation of hSK1 which was paralleled by an increase in sphingosine kinase activity in these cells.

Figure 2 A mutant hSK1 unable to bind TRAF2 is not activated or phosphorylated in response to TNFα. Unlike wild-type hSK1, hSK^{TB2}, a hSK1 mutant defective in its ability to bind TRAF2 [8] is neither activated nor phosphorylated in response to TNFα treatment of HEK293T cells. In contrast, PMA treatment of these cells results in both phosphorylation and activation of wild-type hSK1 and hSK^{TB2}.

Figure 3 Prediction of phosphorylated amino acids in hSK1 using NetPhos. Circled residues represent those amino acids predicted to be phosphorylated in hSK1 (and its mouse and monkey homologues) by the NetPhos program.

Figure 4 Ser^{225} → Ala ablates phosphorylation of hSK1. Metabolic labelling of HEK293T cells transfected with wild-type hSK1, hSK^{S148A}, hSK^{S181A}, hSK^{Y184A}, hSK^{S225A}, hSK^{T250A}, or empty vector and either untreated or treated with PMA showed that only the Ser^{225} → Ala mutation ablated phosphorylation of hSK1.

Figure 5 Ser^{225} → Ala does not effect phosphorylation of hSK1 at adjacent sites. Metabolic labelling of HEK293T cells transfected with wild-type hSK1, hSK^{S220A}, hSK^{S225A}, or hSK^{T222A} and either untreated or treated with PMA again showed that only the Ser^{225} → Ala mutation ablated phosphorylation of hSK1. This indicated that the Ser^{225} → Ala mutation directly effects phosphorylation of hSK1 at Ser^{225} rather than indirectly effecting phosphorylation at adjacent sites (i.e. Ser^{220} and Thr^{222})

Figure 6 hSK^{S255A} is not activated by TNFα or PMA. Unlike wild-type hSK1-transfected HEK293T cells, hSK^{S255A}-transfected HEK293T cells show no increase in sphingosine kinase activity following treatment with TNFα or PMA.

Figure 7 ***In vitro*** **phosphorylation of hSK1.** *In vitro* phosphorylation of purified recombinant hSK1 was examined with ERK1, ERK2 and CDK2. Quantitation of the specific activity of phosphorylation showed ERK2 phosphorylated purified recombinant hSK1 with greatest efficiency.

Figure 8 ***In vitro*** **phosphorylation of hSK1 by ERK2 occurs only at Ser^{225}.** *In vitro* phosphorylation of purified recombinant wild-type hSK1, hSK^{S225A}, hSK^{S148A} and hSK^{T222A} was examined with ERK2. Only hSK^{S225A} was not phosphorylated by ERK2 indicating that ERK2 specifically phosphorylates hSK1 at Ser^{225}.

Figure 9 Increases in hSK1 phosphorylation are blocked by an ERK1/2 pathway inhibitor (PD98059) but not a CDK inhibitor (Olomoucine). Treatment of hSK1-transfected HEK293T cells with 50 μM PD98059 did not effect basal hSK1 phosphorylation, but blocked increases in hSK1 phosphorylation following TNFα or PMA treatment. In contrast, 20 μM Olomoucine appeared to stimulate basal hSK1 phosphorylation which was further increased by PMA treatment.

Figure 10 Activation of hSK1 through ***in vitro*** **phosphorylation by ERK2.** Sphingosine kinase assays of the purified recombinant hSK1 were performed following *in vitro* phosphorylation by ERK2 and compared to untreated purified recombinant hSK1

Figure 11 $Ser^{225} \rightarrow$ Glu in hSK1 does not create a constitutively activated hSK1. hSK^{WT} and hSK^{S225E} were expressed in HEK293T cells and the resultant sphingosine kinase activity in cell lysates quantitated with respect to protein expression levels.

Figure 12 Targets for sphingosine kinase antagonists.

Figure 13 ELISA of phospho-hSK antiserum. Crude (open symbols) and affinity purified (closed symbols) antiserum from rabbits injected with a KLH-conjugated

phosphopeptide designed around Ser^{225} of hSK1 was analysed by ELISA with the phosphopeptide (circles) and corresponding non-phosphorylated peptide (squares).

Figure 14 The phospho-hSK1 antiserum specifically reacts with phosphorylated hSK1 in Western blot analysis. Western blot analysis of hSK1 with the affinity purified phospho-hSK1 antiserum. Lane 1, recombinant hSK1; Lane 2, recombinant hSK1 phosphorylated *in vitro* by ERK2; Lane 3, recombinant hSK1 phosphorylated *in vitro* by ERK2 and subsequently dephosphorylated by alkaline phosphatase.

Figure 15 The phosph-hSK1 antiserum allows phosphorylation of *in vivo* hSK1 to be followed. HEK293T cells were transfected with wild-type hSK1 or hSK^{S225A} and treated with TNFα or PMA, harvested and analysed by Western blot with the phospho-hSK1 antiserum and anti-FLAG antibody.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Specific mutations in amino acid sequence are represented herein as "Xaa_1nXaa_2" where Xaa_1 is the original amino acid residue before mutation, n is the residue number and Xaa_2 is the mutant amino acid. The abbreviation "Xaa" may be the three letter or single letter amino acid code. A mutation in single letter code is represented, for example, by X_1nX_2 where X_1 and X_2 are the same as Xaa_1 and Xaa_2 respectively. In terms of both the mutation and the human sphingosine kinase protein sequence in general, the amino acid residues for human sphingosine kinase are numbered with the residue serine(S) in the motif KT PASPVVVQ being numbered 225.

One aspect of the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

In a preferred embodiment, antagonising said phosphorylation prevents activation of sphingosine kinase and agonising said phosphorylation results in activation of said sphingosine kinase.

Reference to "sphingosine kinase" should be understood as including the reference to all forms of sphingosine kinase protein or derivates, homologues, analogues, equivalents or mimetics thereof. In this regard, "sphingosine kinase" should be understood as being a molecule which is, *inter alia*, involved in the generation of sphingosine 1-phosphate during activation of the sphingosine kinase signalling pathway. This includes, for example, all

protein forms of sphingosine kinase or its functional derivatives, homologues, analogues, equivalents or mimetics thereof, including, for example, any isoforms which arise from alternative splicing of sphingosine kinase mRNA or allelic or polymorphic variants of sphingosine kinase. Preferably, said sphingosine kinase is human sphingosine kinase.

Preferably, this aspect of the present invention is directed to agonising, antagonising or otherwise modulating phosphorylation of human sphingosine kinase at S^{148}, S^{181}, Y^{184}, S^{225} and/or T^{250}. Most prefereably, said phosphorylation is modulated at S^{225}.

In yet another preferred embodiment, said agent modulates the S^{225} sphingosine kinase phosphorylation event which is catalysed by ERK2

Reference to "inducing or otherwise agonising" phosphorylation should be understood as a reference to:

(i) inducing the phosphorylation of sphingosine kinase, for example, inducing the interaction of sphingosine kinase with a proline-directed protein kinase, such as ERK2, which effects sphingosine kinase phosphorylation; or

(ii) up-regulating, enhancing or otherwise agonising an existing phosphorylation event, for example, increasing the affinity of or otherwise stabilising the interaction of sphingosine kinase with a phosphorylating molecule.

Conversely, "inhibiting or otherwise antagonising" phosphorylation should be understood as a reference to:

(i) preventing the interaction of sphingosine kinase with a phosphorylating molecule; or

(ii) antagonising an existing interaction between sphingosine kinase and a phosphorylating molecule such that the phosphorylation of sphingosine kinase is rendered ineffective or less effective.

It should be understood that modulation of the phosphorylation of sphingosine kinase may be partial or complete.

Modulation of the phosphorylation of sphingosine kinase may be achieved by any one of a number of techniques including, but not limited to:

(i) introducing into a cell a proteinaceous or non-proteinaceous agent which antagonises the interaction between sphingosine kinase and a molecule which mediates phosphorylation of said sphingosine kinase;

(ii) introducing into a cell a proteinaceous or non-proteinaceous agent which agonises the interaction between sphingosine kinase and a molecule which mediates phosphorylation of said sphingosine kinase;

(iii) introducing into a cell a proteinaceous or non-proteinaceous agent which phosphorylates sphingosine kinase;

(iv) introducing into a cell a nucleic acid molecule which encodes an agent which phosphorylates sphingosine kinase.

Preferably, said phosphorylation occurs at Ser^{225}.

Reference to "agent" should be understood as a reference to any proteinaceous or non-proteinaceous molecule which modulates (i.e. up-regulates or down-regulates) the interaction of sphingosine kinase with a molecule which phosphorylates sphingosine kinase. "Agent" should also be understood to extend to molecules which, of themselves, phosphorylate sphingosine kinase. The subject agent may be linked, bound or otherwise

associated with any proteinaceous or non-proteinaceous molecule. For example, it may be associated with a molecule which permits targeting to a localised region.

Yet another aspect of the present invention is directed to a method of modulating cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

Preferably, this aspect of the present invention is directed to agonising, antagonising or otherwise modulating phosphorylation of human sphingosine kinase at S^{148}, S^{181}, Y^{184}, S^{225} and/or T^{250}. Most preferably, said phosphorylation is modulated at S^{225}.

In yet another preferred embodiment, said agent modulates the S^{225} sphingosine kinase phosphorylation event which is catalysed by ERK2.

Still another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition characterised by aberrant, unwanted or otherwise inappropriate cellular activity and/or sphingosine kinase activation in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and/or sphingosine kinase and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity and/or sphingosine kinase .

Preferably, this aspect of the present invention is directed to agonising, antagonising or otherwise modulating phosphorylation of human sphingosine kinase at S^{148}, S^{181}, Y^{184}, S^{225} and/or T^{250}. Most preferably, said phosphorylation is modulated at S^{225}.

In yet another preferred embodiment, said agent modulates the S^{225} sphingosine kinase phosphorylation event which is catalysed by ERK2.

Reference to "aberrant, unwanted or otherwise inappropriate" cellular activity should be understood to be understood as a reference to overactive cellular activity, to physiological normal cellular activity which is inappropriate in that it is unwanted or to insufficient cellular activity.

The present invention should also be understood to encompass a method for screening for agents which modulate the phosphorylation of sphingosine kinase, particularly at Ser^{225}, such as agents which agonise or antagonise the interaction of sphingosine kinase with a phosphorylating molecule (such as ERK2) or which themselves phosphorylate sphingosine kinase. This could be achieved, for example, by utilising cell based assays which can monitor sphingosine kinase activation.

Still another aspect of the present invention is directed to agents identified in accordance with the screening method defined herein and to said agents for use in the methods of the present invention. Said agent should be understood to extend to monoclonal antibodies which bind to the phosphorylation sites of sphingosine kinase, and in particular, amino acids S^{148}, S^{181}, Y^{184}, S^{225} and T^{250}.

Yet another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of a medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity and/or sphingosine kinase activity, wherein said agent modulates the phosphorylation of sphingosine kinase and wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and/or sphingosine kinase and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity and/or sphingosine kinase .

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents.

Yet another aspect of the present invention relates to the agents as hereinbefore defined, when use in the method of the present invention.

Still a further aspect of the present invention is directed to sphingosine kinase variants comprising a mutation in a region of said sphingosine kinase comprising a phosphorylation site wherein said variant exhibits ablated or reduced activation capacity relative to wild type sphingosine kinase or a derivative, homologue, analogue, chemical equivalent or pneumatic of said sphingosine kinase variant. The present invention also extends to variants which exhibit enhanced or upregulated activity due to mutations of existing phosphorylation sites or incorporation of additional phosphorylation sites.

Preferably, said variant comprises a mutation at one or more of S^{148}, S^{181}, Y^{184}, S^{225} and/or T^{250} and most preferably at S^{225}. still more preferably, said variant comprises the mutation Ser225Ala.

In yet another aspect, the present invention extends to genetically modified animals, which animals have been modified to express a sphingosine kinase variant as hereinbefore defined.

The present invention is further described by reference to the following non-limiting description.

DETAILED DESCRIPTION OF THE INVENTION

Methods

Generation of phospho-hSK1-specific polyclonal antibodies – Polyclonal antibodies were raised in rabbits against a phosphopeptide (CGSKTPApSPVVVQQ) designed from the hSK1 sequence around Ser^{225}. Prior to injection into rabbits, the phosphopeptide was conjugated to maleimide activated keyhole limpet hemocyanin (Pierce) via its *N*-terminal cysteine. Antibodies active against the non-phosphorylated peptide were removed from the antiserum using SulfoLink™ beads (Pierce) to which the non-phosphorylated peptide (CGSKTPASPVVVQQ) was conjugated using the manufactures instructions.

Construction and expression of hSK mutants – Wild-type human SK1 (hSKWT) cDNA [26] (Genbank accession number AF200328) was FLAG epitope tagged at the 3' end and subcloned into pALTER (Promega Inc., Madison, WI) site directed mutagenesis vector, as previously described [15]. Single-stranded DNA was prepared and used as template for oligonucleotide directed mutagenesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotides shown in Table 1 were designed to generate the required mutants which were subsequently sequenced to verify incorporation of the desired modification. The mutant cDNA were then subcloned into pcDNA3 (Invitrogen Corp., San Diego, CA) for transient transfection into HEK293T cells of pGEX-4T2 for expression as glutathione-*S*-transferase (GST)-fusion proteins in *E. coli*. hSK^{TB2} was generated as previously described [8].

Cell Culture and Transfection – Human embryonic kidney cells (HEK293T, ATCC CRL-1573) were cultured on Dulbecco's modified Eagle's medium (CSL Biosciences, Parkville, Australia) containing 10% fetal calf serum, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and gentamycin (1.6 mg/ml). Transfections were performed using the calcium phosphate precipitation method. Cells were harvested 24 h after transfection and lysed by sonication (2 watts for 30 s at 4 °C) in lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 150

mM NaCl, 1 mM dithiothreitol, 2 mM Na3VO4, 10 mM NaF, 1 mM EDTA and protease inhibitors (Complete™; Boehringer Mannheim). Activation of hSK1 was assessed by treatment of cells with either phorbol 12-myristate 13-acetate (PMA; Sigma) for 30 min, or tumor necrosis factor-α (TNFα; R&D Systems Inc., Minneapolis, MN) for 10 min. Protein concentrations in cell homogenates were determined with Coomassie Brilliant Blue (Sigma) reagent using BSA as standard.

Generation of recombinant proteins in E. coli – Recombinant proteins were generated of in *E. coli* and purified as previously described [26].

Metabolic labelling of cells with ^{32}P – HEK293T cells were cultured and transfected as described above. The cultures, in 10 cm dishes, were incubated for 30 min with phosphate-free, serum-free Dulbecco's modified Eagle's medium (DMEM). This medium was then replaced with fresh phosphate-free, serum-free DMEM containing 500 μCi [^{32}P]orthophosphate and the cultures incubated for 3 hr. Cultures were then stimulated with 1 ng/ml TNF or 100 ng/ml PMA for 30 min. Cells were then washed 3 times with cold PBS and scaped into 1.2 ml of lysis buffer B containing 50 mM Tris/HCl (pH 7.4), 1 % (w/v) Trition X-100, 1 % (w/v) deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 10 mM sodium orthovanadate, 1 mM EDTA, 1 mM DTT, and protease inhibitors (Complete™). Cells were subjected to three freeze-thaw cycles to ensure complete disruption. The cell debris was then removed by centrifugation (13,000 *g* for 20 min at 4°C) and the supernatant incubated with 12 μg of M2 anti-FLAG antibody (Sigma) overnight with mixing at 4°C. The immunocomplexes were then collected with Protein A-Separose and washed four times with lysis buffer B. The immunoprecipitates were then subjected to SDS-PAGE on 12 % acrylamide gels, the gels dried and the phosphorylated hSK quantitated by Phosphorimager (Molecular Dynamics).

In vitro *phosphorylation of hSK1* – Purified recombinant hSK1 (1.5 μg) was incubated with recombinant ERK2 (0.1 μg; Upstate Biotechnology), 125 μM ATP and 5 μCi [γ^{32}P]ATP in 20 mM MOPS (pH 7.2) containing 20 mM $MgCl_2$, 25 mM ß-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT. The

reaction was stopped after 20 min at 37°C by the addition of SDS-PAGE sample buffer, the mixture boiled for 5 min, subjected to SDS-PAGE on 12 % acrylamide gels, the gels dried, and the phosphorylated hSK1 quantitated by Phosphorimager.

Sphingosine kinase assays – SK activity was routinely determined using D-erythro-sphingosine (Biomol, Plymouth Meeting, PA) and [γ32P]ATP (Geneworks, Adelaide, South Australia) as substrates, as described previously [26]. A unit (U) of SK activity is defined as the amount of enzyme required to produce 1 pmol S1P/min.

Results

hSK1 is phosphorylated in HEK293 cells in response to cell agonists – The *in vivo* phosphorylation of hSK1 in HEK293 cells in response to TNFα and phorbol esters (PMA) was examined since phosphorylation is a common mechanism of regulating the catalytic activity of many eukaryotic. Metabolic labelling, with [^{32}P]orthophosphate, of HEK293 cells stably over-expressing hSK1 1000-fold revealed that hSK1 is, indeed, phosphorylated, and that this phosphorylation increases rapidly in response to cell exposure to TNFα (Fig 1) and PMA. Furthermore, this increase in hSK1 phosphorylation is highly correlated with the observed increase in sphingosine kinase activity in these cells in response to TNFα (Fig 1) and PMA. These observations suggested that phosphorylation plays a role in hSK1 activation. Further evidence for this was also obtained using a hSK1 mutant defective in its ability to bind TRAF2. This hSK1 mutant was is neither activated [8] or phosphorylated in response to TNFα (Fig 2). PMA, however, induces both activation [8]and phosphorylation of this mutant (Fig 2).

hSK1 is phosphorylated at Ser^{225} – Analysis of the hSK1 sequence with the NetPhos phosphorylation site prediction program [28] allowed the identification of several possible phosphorylation sites in hSK1 that were also conserved in the mouse and monkey SK1 isoforms (Fig 3). Alanine mutagenesis of these possible sites revealed that the hSK1 mutant containing the Ser^{225} → Ala mutation was not phosphorylated (Fig 4). This suggested Ser^{225} as the sole physiologic phosphorylation site in hSK1. Further mutagenesis

to alanine of Ser^{220} and Thr^{222}, two potentially phosphorylatable amino acids in the region around Ser^{225} had no effect on hSK1 phosphorylation (Fig 5). This indicated that the Ser^{225} → Ala mutation directly blocks phosphorylation at Ser^{225} rather than having a secondary effect on phosphorylation at an adjacent site (i.e. Ser^{220} or Thr^{222}).

Activation of hSK1 requires phosphorylation at Ser^{225} – The hSK^{S225A} mutant, when expressed in HEK293 cells, cannot be activated by cell treatment with TNFα or PMA (Fig 6). This provides strong evidence that phosphorylation Ser^{225} is essential for hSK1 activation.

ERK1, ERK2, and CDK2 specifically phosphorylate hSK1 at Ser^{225} in vitro – The amino acid sequence around Ser^{225} is SKTPAS^{225}PVVVQ. The presence of a proline immediately C-terminal to Ser^{225} suggests a member of the proline-directed protein kinase family is responsible for hSK1 phosphorylation. In particular, the PASP sequence of this region is reminiscent of the ERK1/2 substrate recognition motif, PxS/TP, where x represents a small, neutral amino acid [29]. Experiments examining *in vitro* phosphorylation of purified recombinant hSK1 with ERK1, ERK2 and cyclin-dependent kinase 2 (CDK2) showed that all three kinases can phosphorylate hSK1 (Fig 7). Of these kinases, however, ERK2 showed the greatest efficiency of hSK1 phosphorylation. Further *in vitro* phosphorylation analysis with purified recombinant hSK^{S225A} mutant showed that ERK2 could not phosphorylate this protein (Fig 8). ERK2 was, however, still able to phosphorylate recombinant hSK1 mutants containing alaine mutations the only other potential proline-dependent kinase phosphorylation sites in hSK1 (Ser^{148} and Thr^{222}). This indicated that ERK2 specifically phosphorylates hSK1 at Ser^{225}.

Increases in hSK1 phosphorylation in cells are blocked by a ERK1/2 pathway inhibitor, but not a CDK inhibitor – The *in vivo* phosphorylation of hSK1 in HEK293 cells in response to TNFα and PMA in the presence of PD98059 (a chemical inhibitor of the ERK1/2 pathway) and Olomoucine (a chemical inhibitor of CDKs) was examined. The results (Fig 9) show that PD98059 prevents TNFα- and PMA-induced increases in hSK1 phosphorylation. In contrast, the presence of Olomoucine does not block PMA-induced

hSK1 phosphorylation, and actually appears to increase basal hSK1 phosphorylation in the absence of agonist stimulation. This data suggests the ERK1/2 pathway is necessary for hSK1 phosphorylation.

In vitro *activation of hSK1 by ERK2 – In vitro* phosphorylation of purified recombinant hSK1 at Ser^{225} by ERK2 results in an approximate 4-fold increase in catalytic activity of hSK1 (Fig 10). This suggests that phosphorylation induces a conformational change in the protein structure of hSK1 that directly increases its catalytic efficiency.

Mutagenesis of $Ser^{225} \rightarrow$ Glu does not create a constitutively activated hSK1 – By virtue of their negative charge the acidic amino acids glutamate and aspartate can sometimes mimic phosphorylated amino acids and create a protein conformation resembling the activated state. However, a hSK1 mutant containing the $Ser^{225} \rightarrow$ Glu mutation had similar activity to the wild-type hSK1 (Fig 11). Therefore, unlike the case in some other enzymes this mutation not create a constitutively activated hSK1.

Phospho-hSK1-specific polyclonal antibodies – The crude polyclonal antisera raised in rabbits against the phosphopeptide centred around Ser^{225} of hSK1 was highly reactive in ELISA towards the phosphopeptide, but also showed similar reactivity to the non-phosphorylated peptide (Fig 14). However, following affinity chromatography using the non-phosphorylated peptide to remove reactivity to this peptide, the antiserum showed high specificity in ELISA to the phosphopeptide (Fig 14). Furthermore, high specificity was also shown for the phosphorylated hSK1 protein in Western blots (Fig 15). In these Western blots, the antiserum showed reactivity towards ERK2 phosphorylated hSK1, but showed little reactivity towards the non-phosphorylated wildtype hSK1 (Fig 15) or the hSK^{S225A} mutant (Fig 16). Thus, we have used this purified antiserum to show TNFα and PMA-dependent increases in hSK1 phosphorylation (Fig 16).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also

includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1 Mutagenic oligonucleotides used for site-directed mutagenesis of hSK1

Mutation	Sequence
S148A	CGGCTGCTGGCGCCCATGAAC
S181A	TGTGGACCTCGAGGCTGAGAAGTA
Y184A	AGTGAGAAGGCTCGGCGCCTGGGGG AG
S220A	AAGAGTGGGCGCCAAGACAC
T222A	AAGAGTGGGATCCAAGGCGCCTGCCT CC
S225A	AAGACACCTGCGGCGCCCGTTGTG
S225E	ACACCTGCCGAACCGGTTGTGGTC
T250A	TCTCACTGGGCAGTGGTGC

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Figure 1

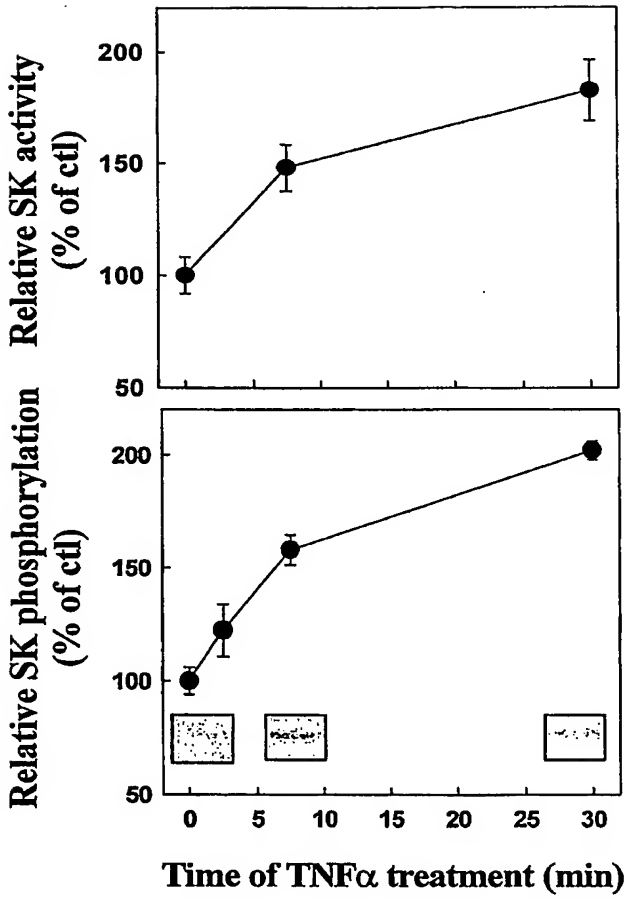
Relative SK activity (% of ctl)
200
150
100
50
Relative SK phosphorylation (% of ctl)
200
150
100
50
0
5
10
15
20
25
30
Time of TNFα treatment (min)

Figure 2

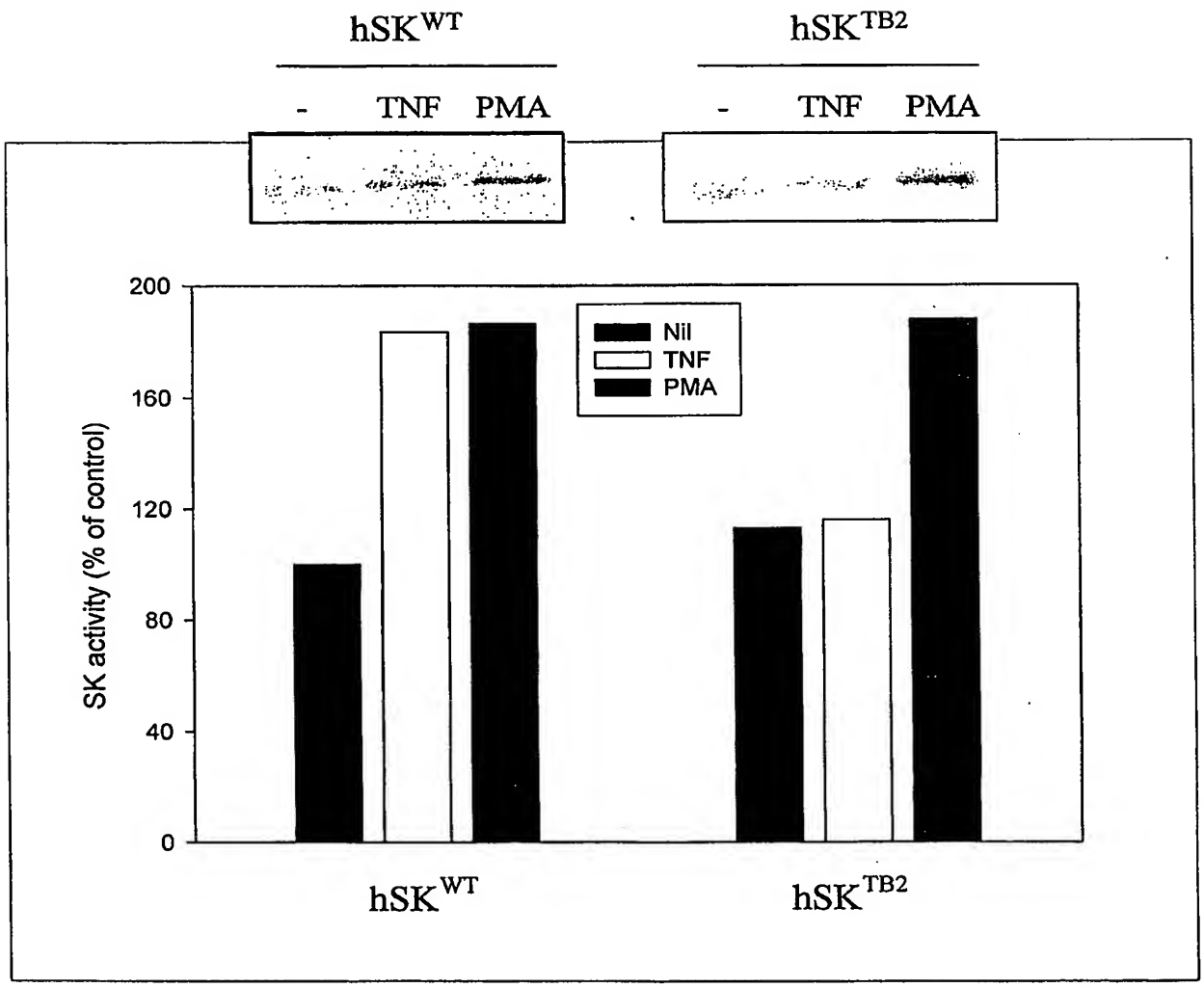
hSK^WT
hSK^TB2
-
TNF
PMA
-
TNF
PMA
200
160
120
80
40
0
SK activity (% of control)
Nil
TNF
PMA
hSK^WT
hSK^TB2

Figure 3

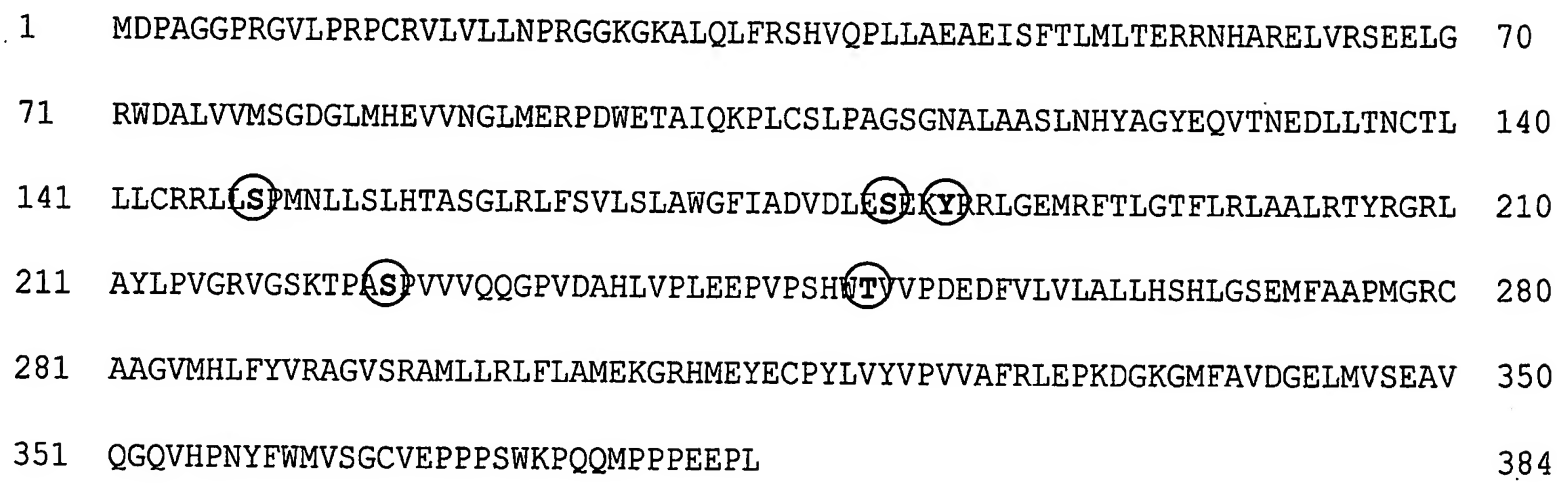
1 MDPAGGPRGVLPRPCRVLVLLNPRGGKGKALQLFRSHVQPLLAEAEISFTLMLTERRNHARELVRSEELG 70
71 RWDALVVMSGDGLMHEVVNGLMERPDWETAIQKPLCSLPAGSGNALAASLNHYAGYEQVTNEDLLTNCTL 140
141 LLCRRLLSPMNLLSLHTASGLRLFSVLSLAWGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGRL 210
211 AYLPVGRVGSKTPASPVVVQQGPVDAHLVPLEEPVPSHWTVVPDEDFVLVLALLHSHLGSEMFAAPMGRC 280
281 AAGVMHLFYVRAGVSRAMLLRLFLAMEKGRHMEYECPYLVYVPVVAFRLEPKDGKGMFAVDGELMVSEAV 350
351 QGQVHPNYFWMVSGCVEPPPSWKPQQMPPPEEPL 384

Figure 4

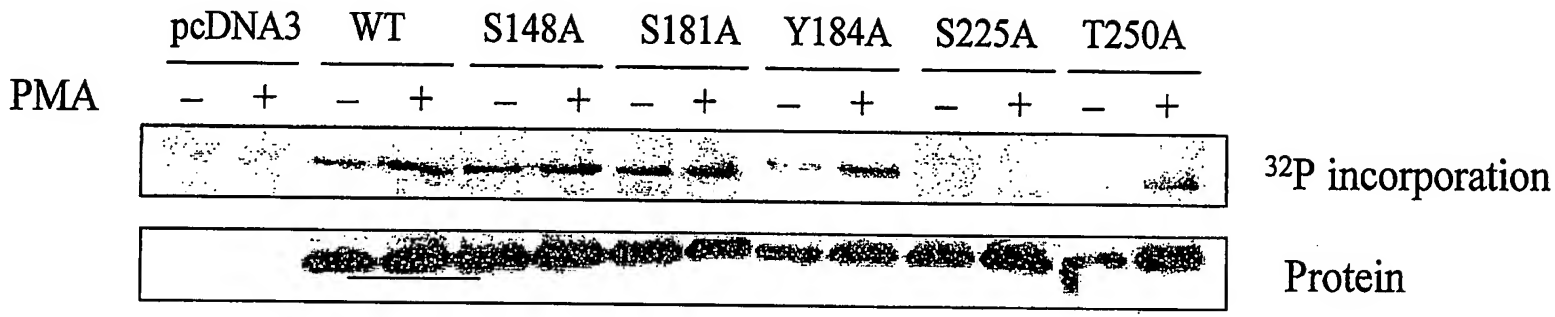
pcDNA3
WT
S148A
S181A
Y184A
S225A
T250A
PMA
– + – + – + – + – + – + – +
32P incorporation
Protein

Figure 5

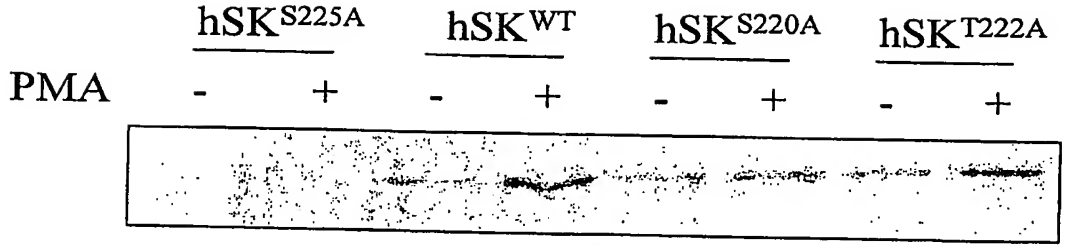

Figure 6

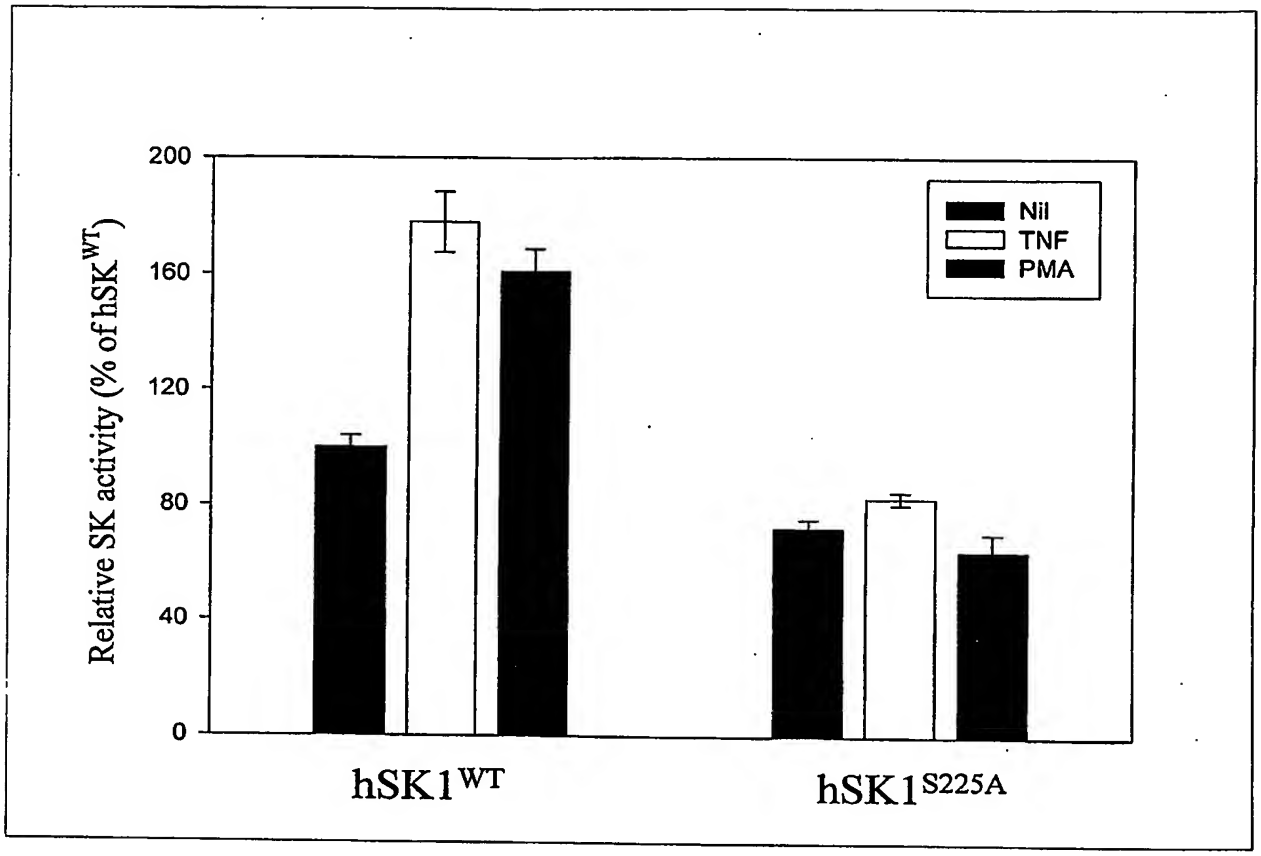
Relative SK activity (% of hSK^WT)
200
160
120
80
40
0
Nil
TNF
PMA
hSK1^WT
hSK1^S225A

Figure 7

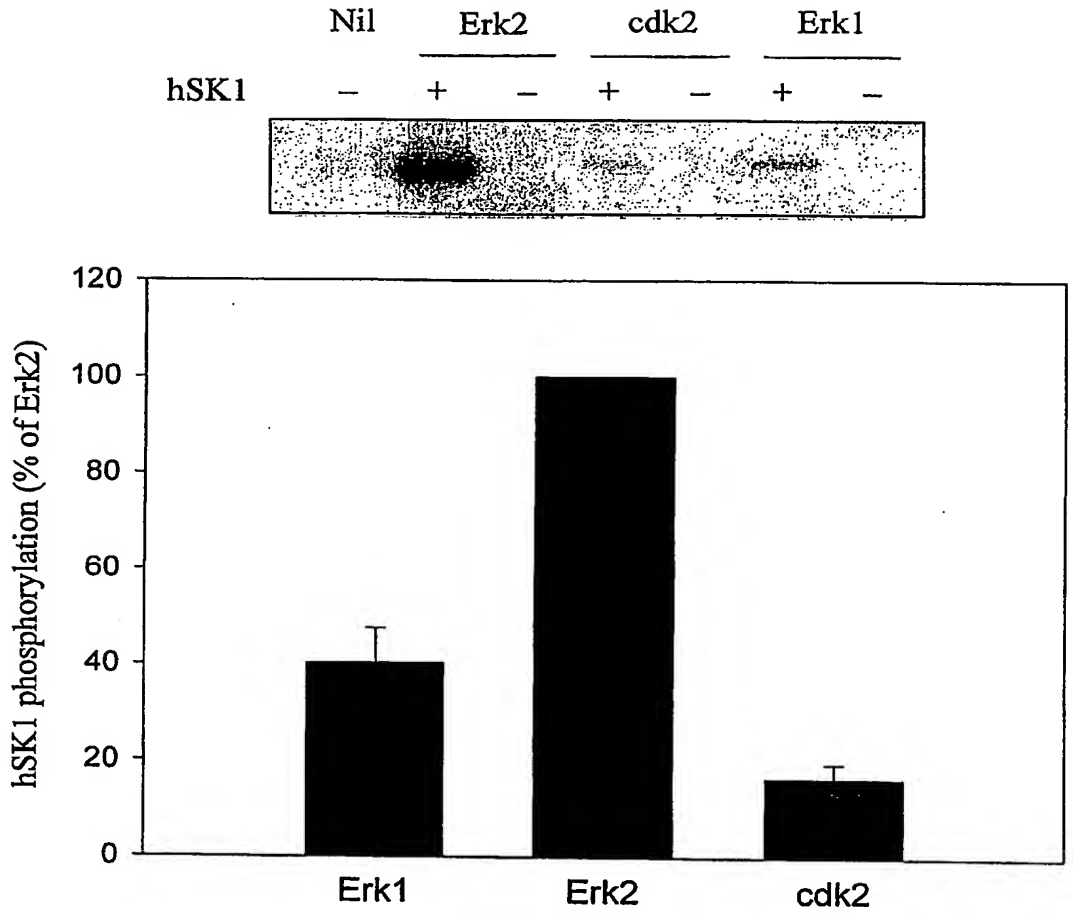
Nil
Erk2
cdk2
Erk1
hSK1
– + – + – + –
hSK1 phosphorylation (% of Erk2)
120
100
80
60
40
20
0
Erk1
Erk2
cdk2

Figure 8

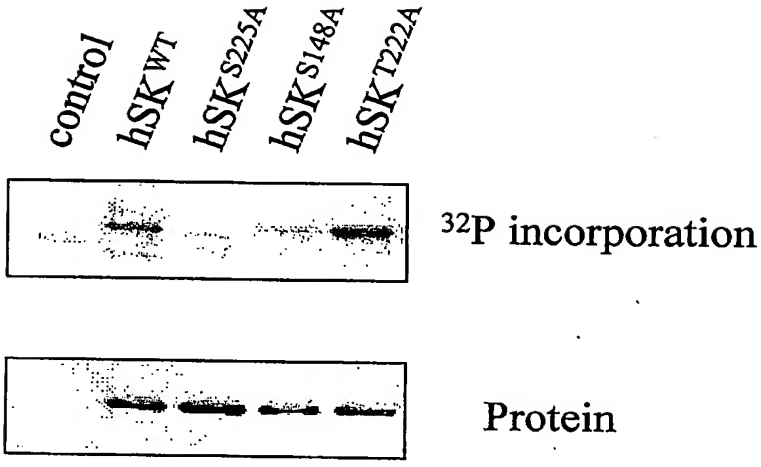
control
hSK WT
hSK S225A
hSK S148A
hSK T222A
32P incorporation
Protein

Figure 9

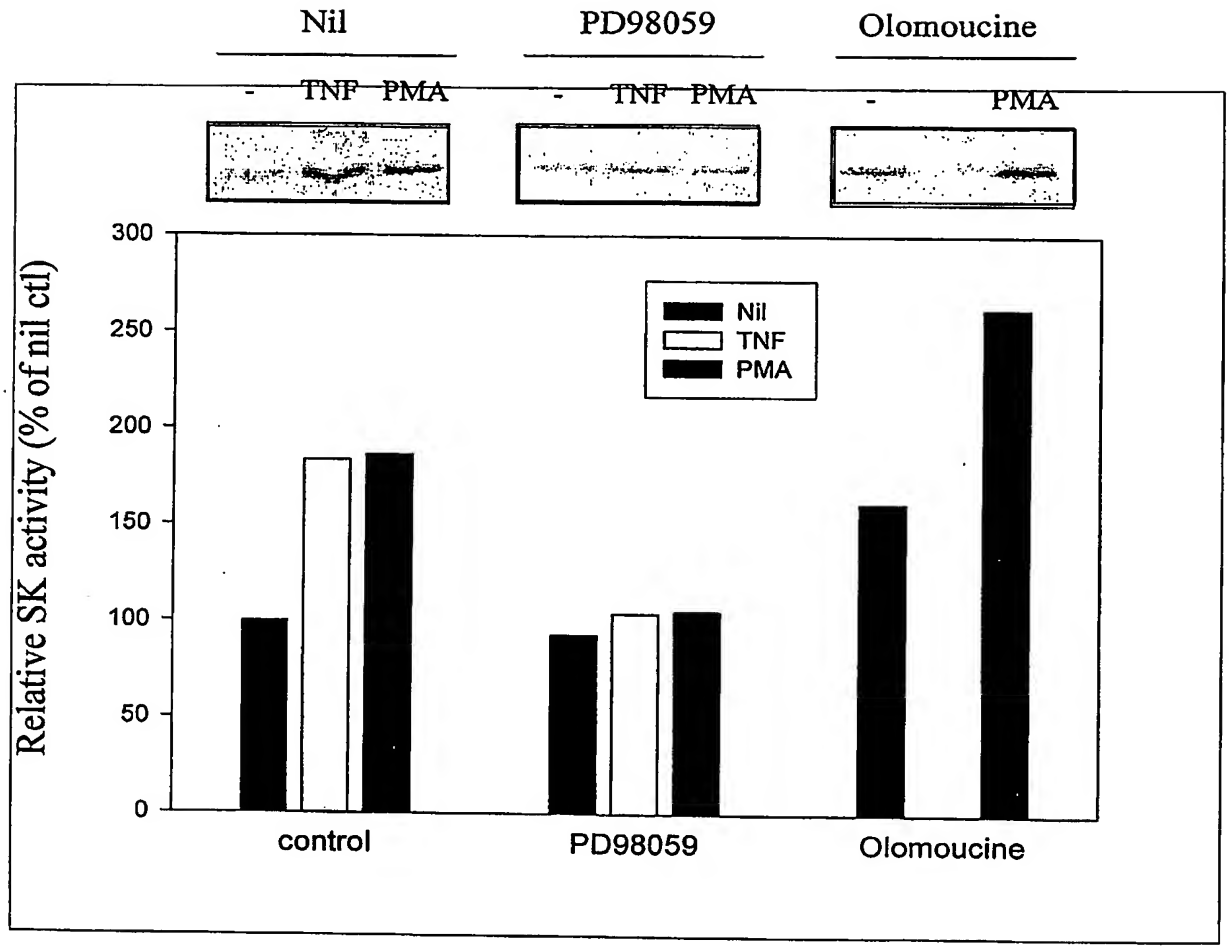
Nil
PD98059
Olomoucine
- TNF PMA
- TNF PMA
- PMA
Relative SK activity (% of nil ctl)
300
250
200
150
100
50
0
Nil
TNF
PMA
control
PD98059
Olomoucine

Figure 10

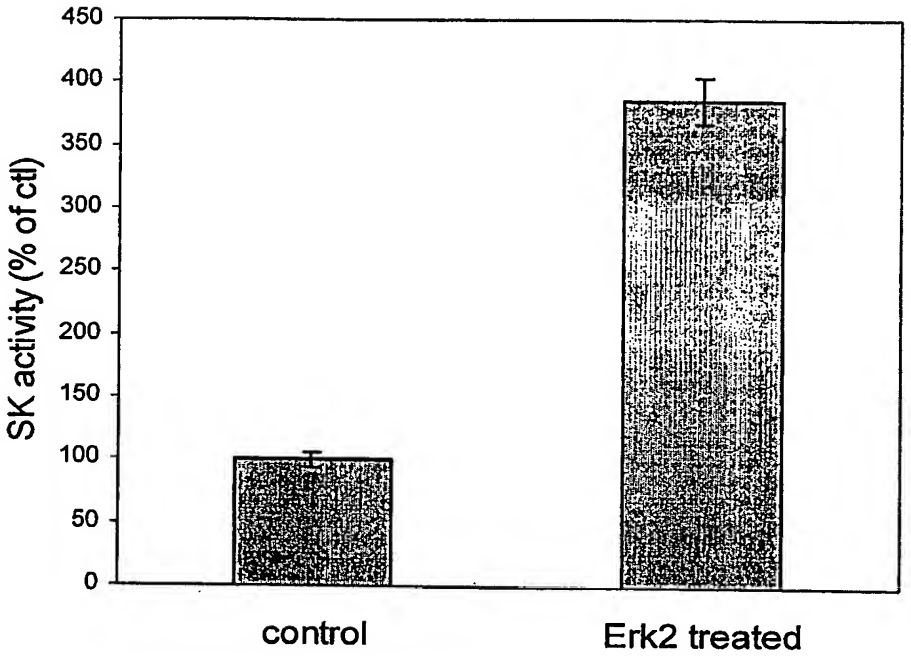
SK activity (% of ctl)
450
400
350
300
250
200
150
100
50
0
control
Erk2 treated

Figure 11

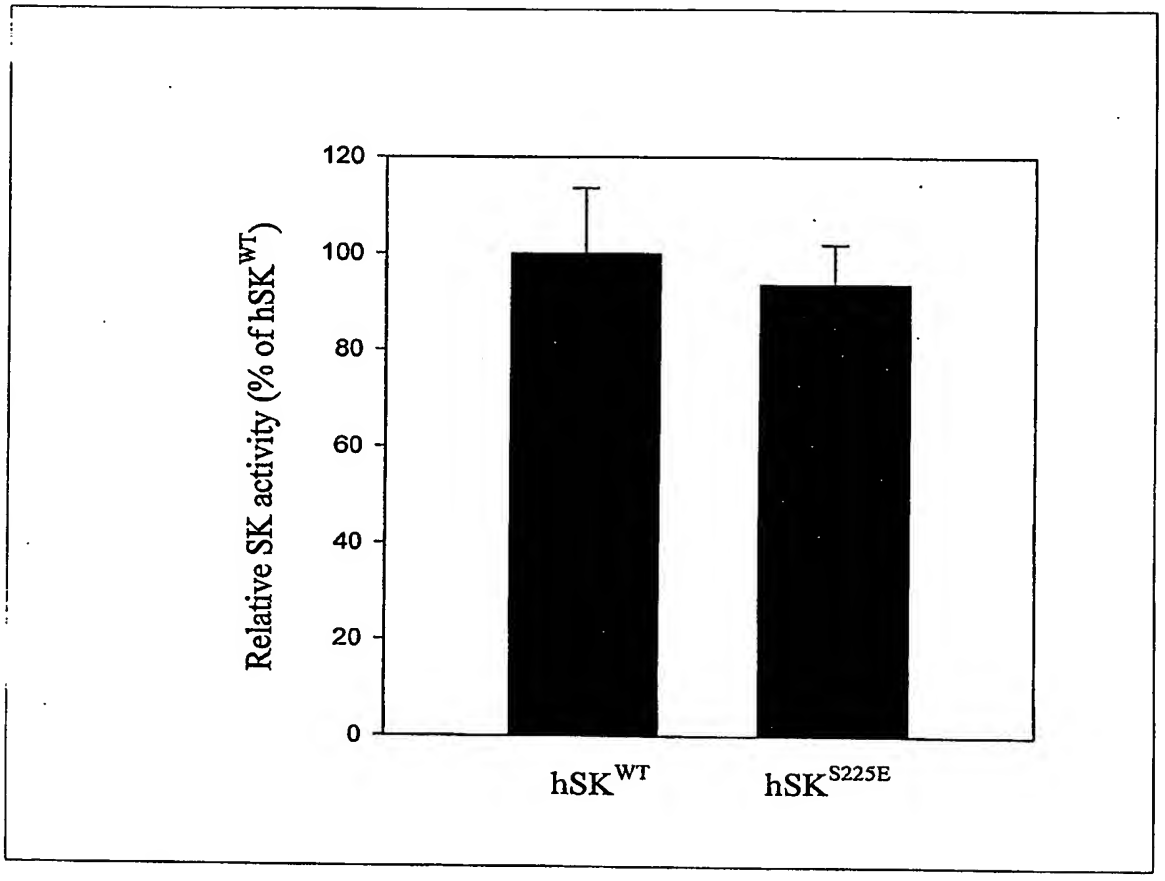
Relative SK activity (% of hSK^WT)
120
100
80
60
40
20
0
hSK^WT
hSK^S225E

Figure 12

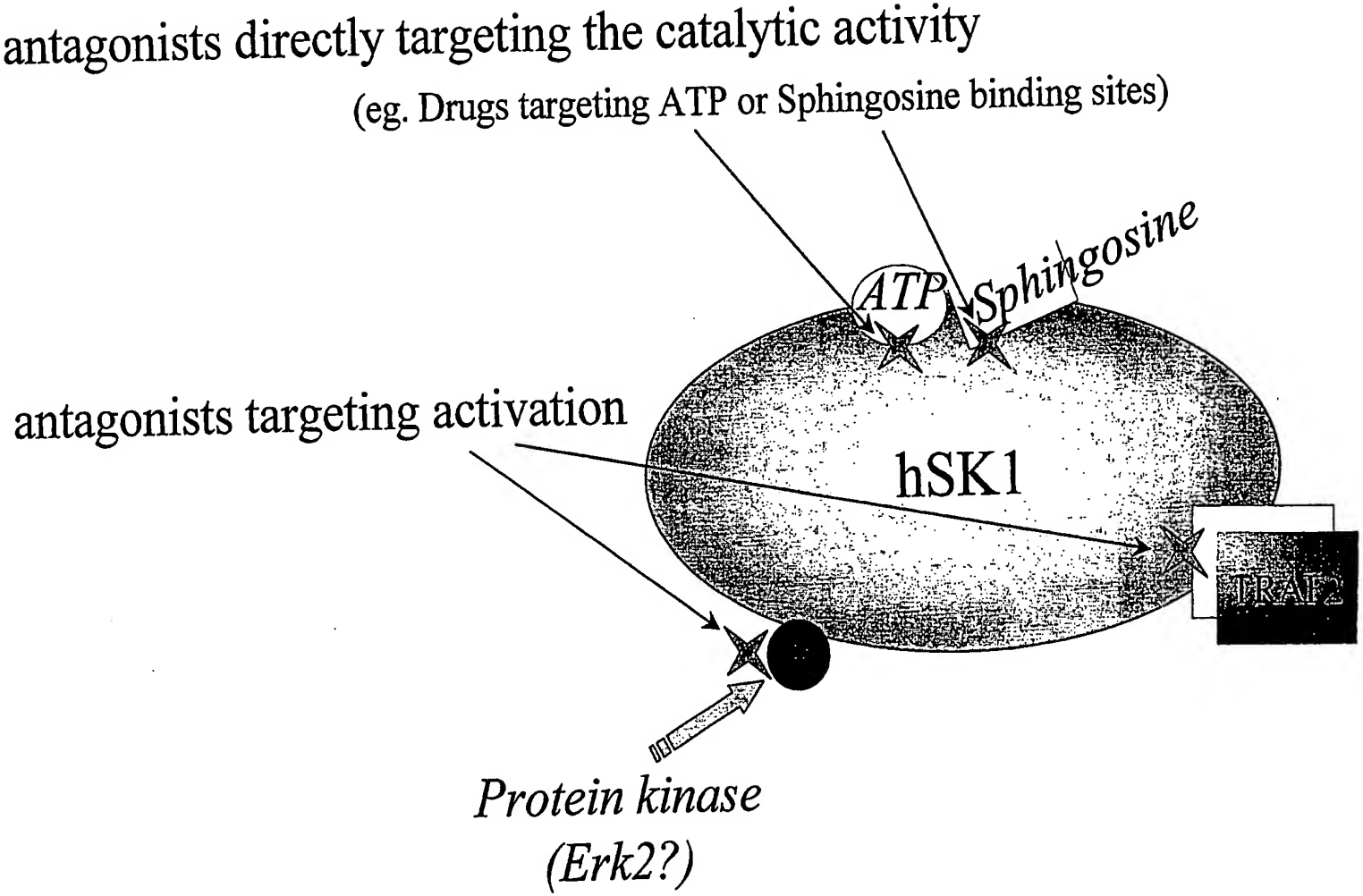
antagonists directly targeting the catalytic activity
(eg. Drugs targeting ATP or Sphingosine binding sites)
ATP
Sphingosine
antagonists targeting activation
hSK1
TRAF2
Protein kinase
(Erk2?)

Figure 13

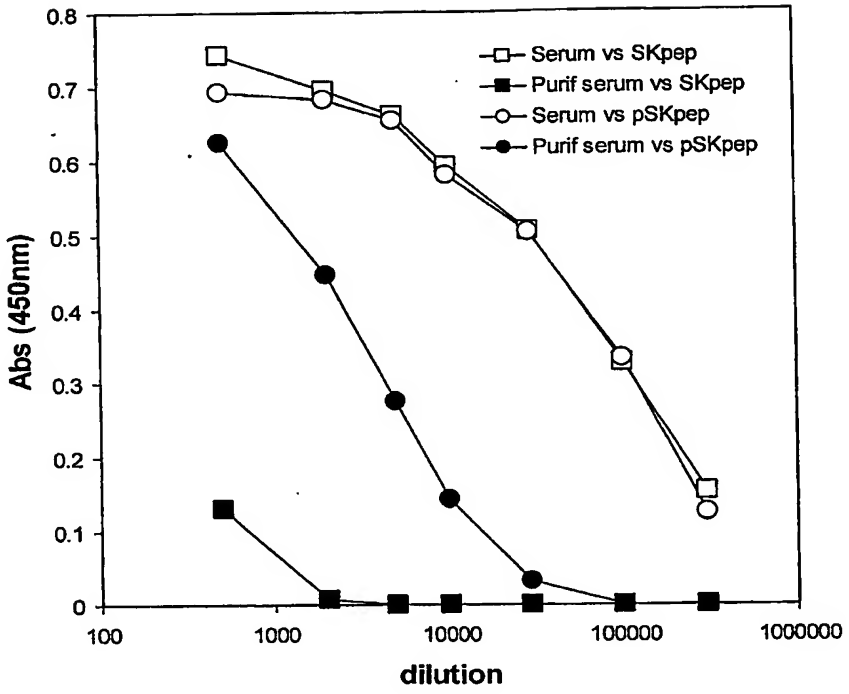
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Abs (450nm)
100
1000
10000
100000
1000000
dilution
Serum vs SKpep
Purif serum vs SKpep
Serum vs pSKpep
Purif serum vs pSKpep

Figure 14

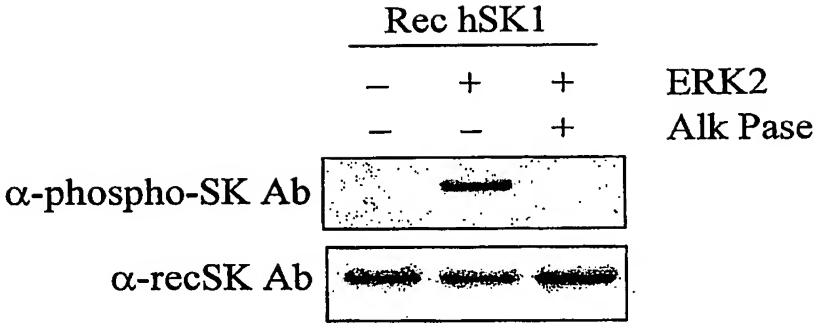

Figure 15